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- 1) orelope et al. proc soc exp biol med 194 / 4 : 301- 307 (1990)
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- 3) horejsi et al. febs letters 288 (n1-2) : 1-4 (1991)

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Alterations in Monoclonal Antibody Affinity and Antigenic Receptor Site Expression on Mycoplasma-Infected Human Colorectal Cancer Cells (43095)

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Abstract. The affinity of MoAb CO 17-1A and expression of its antigenic target were studied on uninfected and mycoplasma-infected colorectal cancer cell lines SW 1116 and SW 948. Binding of ¹²⁵I-labeled CO 17-1A to SW 1116 cells was quantified at 37°C by determination of the affinity constant (K_a) and the number of antigenic receptor sites (r) per cell using Scatchard plots. When mycoplasma-free SW 1116 cells were used as targets, K_a was $0.92 \pm 0.06 \times 10^8 M^{-1}$ and $r = 1.32 \pm 0.14 \times 10^4$ at 37°C. One batch of unspliced, mycoplasma-infected SW 1116 cells had reduced affinity and a decreased number of antigenic receptor sites per cell for ¹²⁵I-labeled 17-1A, while another batch of infected SW 1116 cells had a 4- to 5-fold increase in r and diminished K_a for the antibody compared with uninfected cells. When unspliced, mycoplasma-infected SW 948 cells were exposed to ¹²⁵I-labeled 17-1A and the data subjected to Scatchard analysis, the affinity of the antibody deviated markedly from linearity and rendered analysis for K_a and r meaningless. These data indicate that mycoplasma infection can produce variable effects on the cellular expression of antigenic receptor sites and the affinity of antibody for its target, and emphasize the importance of using mycoplasma-free cell lines in studies of these parameters.

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Infection of cell cultures by mycoplasma, without evident morphologic changes of the contaminated lines, is commonly observed in the *in vitro* propagation of cells under sterile conditions (1, 2). Mycoplasma can affect cellular functions (3-7), interfere with the biosynthesis of macromolecules (8-11), cause chromosomal aberrations, inhibit or stimulate lymphocyte blastogenesis (5, 12), and produce variable cytopathic effects (5, 6). Since mycoplasma infection of human and animal cell lines is commonly undetected, functional assays that define the potential usefulness of newly produced MoAbs can be adversely affected (13-

15). Cultured cells of various lines currently are being used in *in vitro* studies to evaluate immunologic properties of MoAbs or characterize them in terms of their affinities for their targets and numbers of antigenic receptor sites. The affinity constant (K_a) and number of antigenic receptor sites (r) per cell are particularly important since the *in vitro* binding affinity that a radiolabeled MoAb has for cancer cells can be predictive of its potential *in vivo* usefulness for radioimmuno-detection or for targeting tumoricidal agents. The present report describes the heretofore unreported effects of mycoplasma infection on the affinity of MoAbs and receptor site expression on tumor cells. The K_a of MoAb 17-1A was reduced and its antigenic receptor sites per cell were variably increased or decreased on mycoplasma-infected colorectal cancer cells SW 1116 and SW 948.

Materials and Methods

Tumor Cells. Human colorectal cancer cell lines SW 948 and SW 1116 that were used in this study have been described elsewhere (16). They consist of both dedifferentiated and differentiated cells with microvesicular bodies. These cell lines synthesize high levels of

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carcinoembryonic antigen and their chromosome numbers consist of bimodal populations of hypodiploid and hypertriploid cells (16). The cell lines were cultured separately in supplemented Leibovitz's L-15 medium containing 10% fetal bovine serum (FBS). Rat glioma clone F98 was also used in this study. It is an anaplastic glioma derived from an undifferentiated neoplasm transplacentally induced by *N*-ethyl-*N*-nitrosourea in an inbred CD Fischer rat. It has been propagated both *in vitro* and *in vivo* for over 16 years. Its morphology and *in vitro* characteristics have been described in detail (17, 18). This clone was cultured in supplemented Dulbecco's modified Eagle's medium containing 10% FBS.

Detection of Mycoplasma. Contamination of cell cultures by mycoplasma was confirmed by means of the cultivation method (1, 2). Mycoplasma growth media, PPLO agar, and arginine broth were inoculated with equal amounts of cultured cell suspensions in the absence of antibiotics. The broth and agar were then incubated for up to 4 weeks at 37°C in a CO₂ incubator. A change in the pH of the broth and appearance of mycoplasma colonies in agar plates were taken as positive indication of mycoplasma contamination. After 2 weeks of incubation, the agar plates were periodically checked by light microscopy for the presence of mycoplasma colonies, which subsequently were not speciated.

Monoclonal Antibody. Murine MoAb CO-17-1A IgG that was raised against human colorectal carcinoma cells was used in this study. It binds to a nonshedding cell surface membrane antigen expressed by adenocarcinomas of the colon, rectum, stomach, pancreas, and to a lesser extent other human adenocarcinomas (19-22). It also shows weak binding to normal human gastrointestinal tissues (19, 20).

Radioiodination of MoAb CO-17-1A IgG. One milligram of 17-1A was radioiodinated with Na¹²⁵I (1-4 mCi; Medi-Physics, Inc., Tuxedo, NY) by using Iodogen[®], as previously described (23, 24). Free ¹²⁵I was separated from radiolabeled 17-1A by means of Sephadex G25 column chromatography. The ¹²⁵I-labeled 17-1A was further purified with an Amicon Centricon-10 microconcentrator (Amicon Corporation, Danvers, MA) and the percentage of ¹²⁵I bound to the MoAb was assessed by instant thin layer chromatography and trichloroacetic acid precipitation. It was determined by means of these methods that >90% of the ¹²⁵I was bound to the MoAb.

Measurement of Immunoreactivity of ¹²⁵I-Labeled 17-1A against 17-1A. The immunoreactivity of ¹²⁵I-labeled 17-1A against 17-1A antigens on mycoplasma-free SW 948 or SW 1166 cells was determined by means of a previously reported indirect immunofluorescence assay (25) and an enzyme-linked immunosorbent assay (ELISA). For the assessment of immu-

noreactivity by means of ELISA, equal but varying concentrations of unlabeled 17-1A IgG and ¹²⁵I-labeled 17-1A IgG (specific activity ranging from 1 to 4 mCi/mg 17-1A) in unsupplemented Leibovitz's L-15 medium were added separately to quadruplicate samples of 1.0 to 1.5 × 10⁵ viable mycoplasma-free SW 948 or SW 1116 cells in 96-well plates. These were incubated at 37°C for 1 hr, then rinsed three times with phosphate-buffered saline (PBS) before the addition of biotinylated antimouse IgG (Vector Laboratories, Inc., Burlingame, CA), and incubated for 20 min at 37°C. These steps were followed by washing three times with PBS, addition of one drop of Vectastain ABC reagent (Vector Laboratories) to each well, and further incubation at 37°C for 20 min. After rinsing the wells five times with PBS, color for spectrophotometric measurement of MoAb reactivity was developed by adding a solution of 2 mM *o*-phenylenediamine dichloride to each well and read at 490 nm in a Biorad model 2550 EIA reader. Loss of immunoreactivity was assessed by comparing the absorbance values of reactivity of ¹²⁵I-labeled 17-1A with those of unlabeled 17-1A.

In Vitro Binding Studies. Determination of nonspecific binding of ¹²⁵I-labeled 17-1A. This was assessed by means of competitive binding of MoAb ¹²⁵I-labeled 17-1A IgG (specific activity of 1 mCi/mg 17-1A IgG) to mycoplasma-free SW 1116 and SW 948 cells in the absence and presence of unlabeled MoAb 17-1A IgG. mycoplasma-free SW 1116 and SW 948 cell lines were separately harvested by treatment for 15 min with 0.5% trypsin containing 0.53 mM EDTA, washed five times with PBS, and separately resuspended in unsupplemented Leibovitz's L-15 medium. Aliquots of SW 1116 cell suspensions (1 × 10⁷ viable cells) were added into duplicate tubes containing no competitor antibody and into those containing varying concentrations (5 nM-6.7 mM) of competitor antibody (unlabeled MoAb 17-1A IgG) and immediately followed by the addition of 10 nM ¹²⁵I-labeled 17-1A IgG into each tube. The assay mixture was brought up to 1 ml with unsupplemented L-15 medium and the contents were gently mixed. The samples were incubated at ambient temperature for 1 hr. Mycoplasma-free F98 glioma cells, which do not express the antigen detected by MoAb 17-1A, were used as a negative control for assessing background binding which routinely was <1.5% of the amount of ¹²⁵I-labeled 17-1A added to 1 × 10⁷ viable F98 cells. At the end of incubation, the cells were washed three times at 4°C with a mixture of 20 ml of McCoy's 5A medium containing 30% FBS and 100 ml of Hanks' balanced salt solution. The amount of ¹²⁵I-labeled 17-1A bound to the cells was determined by counting the cell pellets in a Tracor Analytic model 1185 gamma scintillation counter. Replicate samples showed <1% variation. The background binding was subtracted from total binding obtained in the absence and presence of excess unlabeled 17-1A.

beled MoAb 17-1A IgG. Nonspecific binding of ^{125}I -labeled 17-1A IgG to mycoplasma-free SW 948 cells was similarly studied as described above.

Scatchard analysis. The affinity constant (K_a) and number of receptor sites (r) per cell were quantified according to the method of Scatchard (26). Mycoplasma-infected SW 1116 and SW 948 cell lines were harvested separately from culture and washed five times with PBS. Each cell line was separately suspended in unsupplemented Leibovitz's L-15 medium without FBS and antibiotics. Varying concentrations (0.4–8 μg) of ^{125}I -labeled 17-1A (specific activity of 1 mCi/mg 17-1A IgG) were added to duplicate samples of 1×10^7 viable mycoplasma-infected SW 1116 and SW 948 cells. The cell lines were separately incubated at 37°C for 1 hr. Rat F98 glioma cells were used as a negative control for assessing background binding. Mycoplasma-free SW 1116 cells were used as negative controls and studied as detailed above to differentiate the effects of mycoplasma infection. At the end of incubation, the cells were washed three times at 4°C and cell pellets were counted as described above. Nonspecific and background binding were subtracted from total binding. Replicate samples showed $<1\%$ variation.

Dilution binding studies. These experiments were carried out by incubating duplicate samples of 1×10^7 viable mycoplasma-contaminated SW 1116 cells with a fixed amount of total antibody (^{125}I -labeled 17-1A IgG (specific activity of 1 mCi/mg 17-1A IgG) + 17-1A IgG) and varying the ratio of radiolabeled to unlabeled MoAb. The assay volume was 1 ml and the samples were incubated at 37°C for 1 hr. At the end of the incubation period, the samples were washed three times at 4°C and cell pellets were counted as described above. Rat F98 cells were used to assess background binding, and noninfected viable SW 1116 cells were studied as described above to differentiate the effects of mycoplasma infection. Nonspecific and background binding were subtracted from total binding. Also, replicate samples showed $<1\%$ variation.

Results

Assessment of Immunoreactivity of ^{125}I -Labeled 17-1A. Figure 1 shows the *in vitro* immunoreactivity standard curve, as quantified by ELISA, when unlabeled 17-1A and ^{125}I -labeled 17-1A were reacted with mycoplasma-free SW 1116 cells. When various specific activities ranging from 1 to 4 mCi/mg of 17-1A IgG were used, there was no loss in immunoreactivity of the labeled antibodies compared with unlabeled MoAb (Fig. 1). Moreover, indirect immunofluorescence assays showed that at these levels of specific activity, radioiodination did not significantly alter the recognition of antigen identified by 17-1A and expressed on the cell surface of mycoplasma-free SW 1116 cells (data not shown).

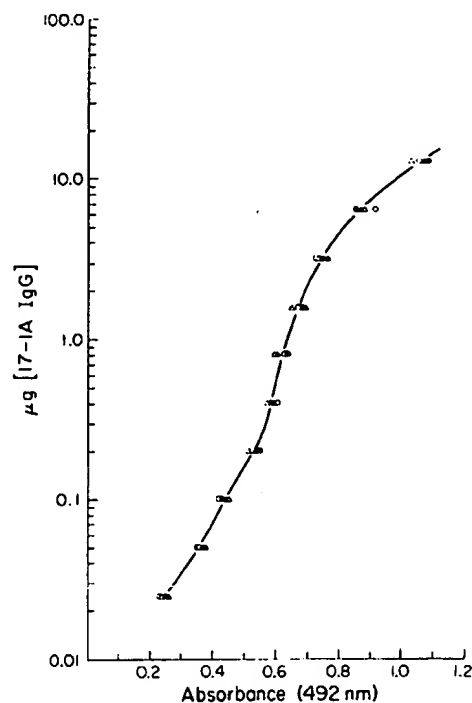


Figure 1. The *in vitro* immunoreactivity standard curve, quantified by ELISA, using the same concentrations of unlabeled (●—●) and ^{125}I -labeled MoAb 17-1A IgG (1 mCi/mg 17-1A, ▲—▲; 3.25 mCi/mg 17-1A, ○—○; 4 mCi/mg 17-1A, △—△) and mycoplasma-free SW 1116 cells as targets. The data indicate no loss of immunoreactivity of the radiolabeled antibody.

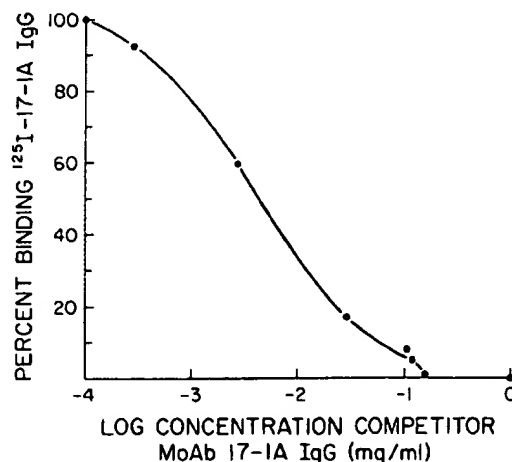


Figure 2. Competitive binding of MoAb ^{125}I -labeled 17-1A IgG and unlabeled 17-1A IgG to mycoplasma-free SW 1116 cells. Duplicate samples of SW 1116 cells were incubated with 10 nM ^{125}I -labeled 17-1A and various dilutions of unlabeled 17-1A. Replicate samples showed $<1\%$ variation.

In Vitro Binding Studies. The competitive binding of labeled and unlabeled MoAb 17-1A on mycoplasma-free SW 1116 and SW 948 cells was examined. As shown in Figure 2, the binding of ^{125}I -labeled 17-1A to SW 1116 cells was completely blocked by unlabeled 17-1A; therefore, nonspecific binding was 0%. Similar

results were obtained with mycoplasma-free SW 948 cells. Furthermore, data from this study demonstrate that both labeled and unlabeled 17-1A recognized and bound to the same antigenic receptor sites on these cell lines and that the binding was specific. There was a linear relationship between binding to mycoplasma-free SW 1116 cells and increasing concentrations of MoAb ^{125}I -labeled 17-1A (represented by open circles in Fig. 3). When the data shown in Figure 3 were subjected to Scatchard analysis, the lines shown in Figure 4 were obtained. With mycoplasma-free SW 1116 cells, MoAb 17-1A IgG detected $1.32 \pm 0.14 \times 10^6$ antigenic receptor sites/cell, and bound with an affinity of $0.92 \pm 0.06 \times 10^8 \text{ M}^{-1}$ at 37°C (Table I and represented by open circles in Fig. 4). In contrast to these findings, when a series of experiments was performed with mycoplasma-infected SW 1116 cells, the following data, summarized in Table I and Figures 3 and 4, were obtained. There was nonlinearity between the binding of these cells and increasing concentrations of ^{125}I -labeled 17-1A IgG at 37°C (represented by closed circles in Fig. 3). There was a reduction in the number of antigenic receptor sites recognized by MoAb 17-1A, as evidenced by decreased values of $>25\%$ for r at 37°C

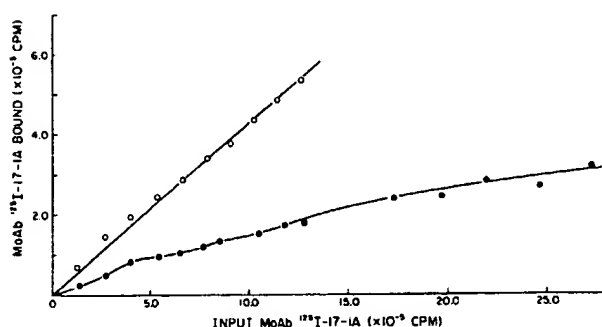


Figure 3. The amount of ^{125}I -labeled 17-1A bound to cells was plotted as a function of input. Mycoplasma-free SW 1116 cells (○—○) and first batch of mycoplasma-infected SW 1116 cells (●—●). Replicate samples showed $<1\%$ variation.

and a decrease in affinity of these cells for MoAb 17-1A (Table I and represented by closed circles in Fig. 4).

Alteration in the number of antigenic sites recognized by MoAb 17-1A could have occurred either as a consequence of radioiodination or masking of antigenic receptor sites by mycoplasma. Although there was no loss in immunoreactivity of ^{125}I -labeled 17-1A, as quantified by ELISA, this possibility, along with the masking of antigenic receptor sites, was studied by means of dilution binding studies. When mycoplasma-free SW 1116 cells were used, the data showed that the binding of ^{125}I -labeled 17-1A to the cells was linearly proportional to the amount of ^{125}I -labeled 17-1A added to the cells, and that there were no differences in the behavior of radiolabeled and unlabeled MoAb 17-1A (represented by open circles with a correlation coefficient of 0.9992 in Fig. 5). The latter supports the findings obtained by ELISA. When mycoplasma-infected SW 1116 cells were studied, the data showed nonlinearity between the amount of ^{125}I -labeled 17-1A added and that bound to the cells, at nonsaturating levels of antibody (represented by closed circles in Fig. 5). This suggests the possibility of masking or alteration of 17-1A antigenic receptor sites by mycoplasma and would explain the reduction at nonsaturating concentrations of the antibody in the binding of these cells to ^{125}I -labeled 17-1A as shown in Figure 3.

When another batch of mycoplasma-infected SW 1116 cells was studied, and the data, after subtracting background and nonspecific binding, were subjected to Scatchard analysis, a line drawn through closed squares in Figure 4 was obtained. The number of antigenic receptor sites per cell detected by MoAb 17-1A increased 4- to 5-fold over those obtained with uninfected cells, but the affinity for 17-1A antigen was reduced by more than 80% (Table I). These observations suggest a masking of the cell surface membrane antigen recognized by MoAb 17-1A and/or expression of mycoplasma epitopes that were similar to those recognized by 17-1A, but with lower or altered affinity. The apparent increase in the number of receptor sites per cell

Table I. Changes in the Affinity of MoAb 17-1A to Mycoplasma-Infected SW 1116 Human Colorectal Cancer Cells

Cell Line ^a	Affinity (K_a) receptors (r)	Temperature of Incubation ^b (37°C)	Comment
SW 1116 (mycoplasma-infected cells)	K_a	$5.80 \pm 0.25 \times 10^7 \text{ M}^{-1c}$	First batch
	r	$0.87 \pm 0.19 \times 10^{8c}$	
SW 1116 (mycoplasma-infected cells)	K_a	$1.04 \pm 0.20 \times 10^7 \text{ M}^{-1c}$	Second batch
	r	$6.28 \pm 0.30 \times 10^{8c}$	
SW 1116 (mycoplasma-free cells)	K_a	$0.92 \pm 0.06 \times 10^8 \text{ M}^{-1}$	
	r	$1.32 \pm 0.14 \times 10^6$	

^a K_a and r could not be determined for Mycoplasma-infected SW 948 cells due to the marked variability in the binding of radiolabeled antibody.

^b Values represent mean \pm SE of five to six experiments.

^c Student's t test (two-tailed analysis): $P < 0.05$ compared with mycoplasma-free cells.

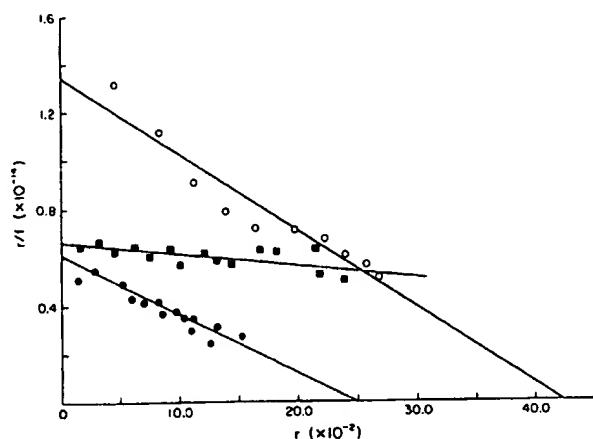


Figure 4. Varying concentrations of MoAb ^{125}I -labeled 17-1A IgG were added to duplicate samples of 10^7 viable mycoplasma-free SW 1116 cells (○—○), 10^7 viable mycoplasma-infected SW 1116 cells (first batch, ●—●), and 10^7 viable mycoplasma-infected SW 1116 cells (second batch, ■—■), and then incubated for 1 hr. The data were subjected to Scatchard analysis (correlation coefficients of -0.9571 (○—○), -0.9359 (●—●), and -0.9006 (■—■), r , nanograms of ^{125}I -labeled antibody bound to 10^7 cells; f , molar concentration of free ^{125}I -labeled antibody).

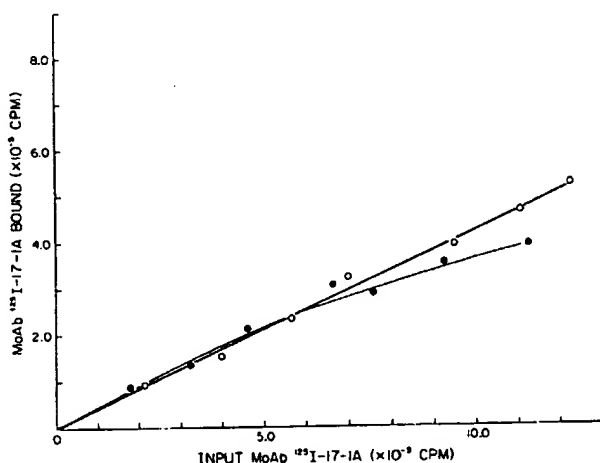


Figure 5. Duplicate samples of mycoplasma-free SW 1116 cells (10^7 cells/sample, ○—○) and of first batch of mycoplasma-infected SW 1116 cells (10^7 cells/sample, ●—●) were incubated for 1 hr at 37°C with a constant amount of protein ($3.2 \mu\text{g}/\text{sample}$) but with varying ratios of ^{125}I -labeled 17-1A IgG and unlabeled 17-1A IgG. The amount bound in cpm was plotted as a function of ^{125}I -labeled 17-1A cpm added to cells. Replicate samples showed $<1\%$ variation. Mycoplasma-free SW 1116 cells linearly bound the antibody with a correlation coefficient of 0.9992 .

may have been due to cross reactivity between mycoplasma antigens and the antigen recognized by 17-1A. Although the mycoplasma that infected these cells were not speciated, nevertheless there is a suggestion that this, as well as the severity of infection, may be important. All of these taken together might explain the findings we obtained from mycoplasma-infected SW 948 cells that showed so much variability that analysis

for K_d and r values would have been meaningless (data not shown).

Discussion

MoAb 17-1A currently is being used for radioimmunodetection or therapy of colon and other gastrointestinal cancers, either alone or as a conjugate with cytotoxic compounds (19, 23, 27–30). It can potentially be used in radioimmunotherapy against tumors that elaborate 17-1A antigen. For example, ^{125}I -labeled 17-1A has been shown to elicit specific cellular cytotoxicity and decreased cell survival following its internalization into SW 1116 cells (31). The degree of internalization was quantified and found to increase during a 48-hr incubation to 49% (31). Also during this incubation period, significant chromosomal aberrations were observed in SW 1116 cells due to ^{125}I Auger electrons, and these damages were not observed when Na ^{125}I , an indifferent radiolabeled MoAb, or cells which do not contain the requisite antigen were used (31). It has been reported to have a K_d of $0.7 \pm 0.1 \times 10^8 \text{ M}^{-1}$ and a r value of 1.0×10^6 when SW 948 cell line was used as the target (32). In the present study, we obtained a K_d of $0.92 \pm 0.06 \times 10^8 \text{ M}^{-1}$ and an r value of $1.32 \pm 0.14 \times 10^6$ when a mycoplasma-free SW 1116 cell line was used as the target. These values are quite close to those obtained with SW 948 cells.

Since Robinson *et al.* (33) first isolated mycoplasma from a contaminated HeLa cell culture, subsequent studies have shown that mycoplasmas are a diverse group of organisms capable of altering cell functions by multiple mechanisms (5). The interaction between most human and animal mycoplasma and host cells is typically through surface adherence. The receptors for some of these organisms are host membrane sialoglycoconjugates or proteins, while for others the chemical nature of the receptors is unknown (5). It is conceivable that the decreased expression of antigenic receptor sites per cell observed in our first study with mycoplasma-infected cells might have been due to membrane protein modifications affected on 17-1A antigen by these organisms. This antigen is a cell surface glycoprotein with N -linked oligosaccharide chains, and is sensitive to proteinase K but not to neuraminidase digestion (34). Some mycoplasma species, for example, *Mycoplasma hyorhinis* and *Mycoplasma fermentans*, are avid cytabsorbing agents and as many as 10^3 organisms of these species have been observed to attach to one infected cell in culture (5). Although the mycoplasma that infected the cell lines in the present study were unspciated, it is possible that the apparent increased expression of antigenic receptor sites per cell observed in the second study of infected cells could have been due to a combination of cellular 17-1A antigenic sites and crossreactive mycoplasma antigens

of each avid cytabsorbing organism similar to *M. hyorhinis* and *M. fermentans*.

There is voluminous literature on the interaction between mycoplasma and cells cultured *in vitro*. To the best of our knowledge, however, no one has attempted to quantify changes in receptor site expression and the affinity of MoAbs to mycoplasma-infected cancer cells. Since mycoplasma contamination of cell cultures may go unnoticed because large numbers of these organisms do not produce overtly turbid media commonly associated with bacterial and fungal contamination, our data emphasize the importance of periodic monitoring of cell lines to rule out mycoplasma contamination. Investigators should be cognizant of the possibility of mycoplasma contamination in order to avoid misinterpretation of data relating to antibody affinity and antigenic receptor site expression.

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